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Emergence of pfhrp2 and pfhrp3 Gene Deletions in Plasmodium falciparum and Diagnostic Challenges in Bangladesh

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Abstract

Background

Histidine-rich protein 2 (HRP2)-based rapid diagnostic tests (RDTs) have been a major tool in the malaria elimination efforts, but their use is complicated by false-negative results in regions where *Plasmodium falciparum* parasites have deletions in the *pfhrp2* and *pfhrp3* genes. But information about the prevalence and significance of these deletions is still limited in Bangladesh. Given the recent reports of diagnostic discordant *P. falciparum* infections from various locations in the malaria-endemic district of Bandarban, which has the highest malaria burden in Bangladesh, we sought to confirm *pfhrp2* and *pfhrp3* gene deletions in suspected samples.

Methods

A descriptive study was carried out in the laboratory with clinical samples of *P. falciparum* from Bandarban from 2023 to 2025. Twenty-two samples from symptomatic persons who were negative by routine HRP2-based RDTs, but positive by microscopy and Plasmodium species-specific polymerase chain reaction (PCR). Microscopy was used to determine parasite densities. The deletion of *pfhrp2*, *pfhrp3* and selected flanking loci were determined by conventional PCR and confirmed by probe-based real-time PCR.

Results

Microscopic examination revealed parasite densities ranging from 440 to 215,000 parasites/μL. Conventional PCR demonstrated *pfhrp2* gene deletion in all isolates, with 20 (90.9%) of 22 also exhibiting *pfhrp3* deletion, indicating a predominance of double-deletion parasites. Probe-based real-time PCR also confirmed *pfhrp2* deletion in all isolates, while only two samples retained detectable *pfhrp3* signals. Analysis of flanking regions revealed that genomic deletions extended beyond the target genes, with conserved loss patterns at the *pfhrp2* locus.

Conclusions

This study shows significant *pfhrp2* gene deletions and frequent co-deletion of *pfhrp3* in *P. falciparum* isolates from Bandarban, with large genomic deletions in the flanking regions. These findings provide the most likely explanation for the observed false-negative HRP2-based RDT results and signal a potentially major threat to the current malaria diagnostic approaches in this high-endemic region. A targeted survey for *pfhrp2/3* deletions is needed to assess the scale and scope of the threat in parallel with careful consideration for alternative interim and long-term diagnostic strategies to ensure accurate case detection and to support effective malaria elimination efforts in Bangladesh.

Introduction

Despite significant progress in malaria incidence over the last 20 years [1] malaria remains a major public health problem, with an estimated 282 million cases and 610,000 deaths in 2024 in the world [2]. *Plasmodium falciparum* (*P. falciparum*) is the most important species of malaria in terms of morbidity and mortality worldwide, among the five species that infect humans [3]. Effective malaria surveillance, prevention and control are impossible without accurate diagnosis. In this regard, microscopy has long been considered the reference standard for diagnosis of clinical malaria [3, 4]. Its performance, however, is very much dependent on the expertise, experience and proper training of the operators, as well as the laboratory infrastructure, which results in inconsistent performance, especially in rural and hard to reach areas [5, 6]. Consequently, rapid diagnostic tests (RDTs) have become the preferred field-deployable diagnostic tool in many low- and middle-income countries due to their ease of use, limited infrastructure requirements, without any requirement of electricity and rapid turnaround time to provide prompt results [5, 7, 8].

Immunochromatographic assays, called Malaria RDTs (mRDTs), are widely used for point-of-care diagnosis and are able to detect parasite antigens in blood [9]. Since the policy change in 2010 by the World Health Organization (WHO) to move towards a universal test-and-treat approach, the uptake of mRDTs has grown significantly and they are now widely deployed across the globe, greatly enhancing the capacity for malaria diagnosis and appropriate case management [10–13]. The majority of these mRDTs detect the presence of *P. falciparum* and are based on the detection of either the histidine-rich protein 2 (PfHRP2) or the Plasmodium lactate dehydrogenase (pLDH) or aldolase enzymes. Despite pLDH and aldolase being conserved in all *Plasmodium* species, PfHRP2-based mRDTs generally have a better analytical sensitivity to *P. falciparum* and are more heat stable [14, 15]. But antibodies used in PfHRP2-binding mRDTs have multiple common epitopes with the structurally similar histidine-rich protein-3 (PfHRP3), with important implications for diagnostic performance [16, 17].

The accumulating evidence over the past few years suggests that there is an alarming global trend, in which *P. falciparum* parasites with deletions in the *pfhrp2* and/or *pfhrp3* genes are increasingly becoming prevalent in malaria-endemic areas [10, 12]. The lack of these target antigens undermines the functionality of HRP2-based mRDTs, leading to false-negative diagnosis and making it a major challenge to malaria surveillance and control activities [12]. The deletions in *pfhrp2/pfhrp3* were initially reported in the Peruvian Amazon in 2010 [5] and have since been documented across a wide geographic range, including numerous countries in South America, Africa, and Asia [10, 13, 17, 18].

Bangladesh in particular has much at stake in this global scenario. Through concerted efforts by the National Malaria Elimination Programme (NMEP) and with the assistance from the Global Fund to Fight AIDS, Tuberculosis and Malaria (GFATM), Bangladesh has been able to make a dramatic decrease in malaria burden over the past decade [19]. Malaria is endemic in 13 districts, mostly in the forested Chittagong Hill Tracts (CHT) bordering India and Myanmar [20, 21]. Bangladesh has been steadily marching towards the goal of malaria elimination by cutting malaria cases by 93% between 2008 and

2020 based on the significant gains made in malaria control [20]. During this phase of declining transmission, however, sporadic cases have been documented in which patients with clinical features of malaria were detected as positive by microscopy but initially tested negative in mRDTs. The first documented indication of such diagnostic discordance was reported in 2017, when a symptomatic *P. falciparum* infection tested repeatedly negative by HRP2-based RDTs despite confirmation by microscopy and molecular assays. Subsequent genetic analysis revealed a partial deletion of the *pfhrp2* gene along with frameshift mutations affecting both *pfhrp2* and *pfhrp3*, providing the first peer-reviewed evidence of HRP2-associated diagnostic escape in Bangladesh [22]. Field-level reports thereafter frequently suggested that similar instances of diagnostic discordance were being encountered. However, systematic documentation and targeted molecular surveillance were not initially undertaken. Over the past few years, we have found more cases of *P. falciparum* with similar discordant diagnostic outcomes. These findings led to a systematic study to identify whether such diagnostic failures could be due to genetic changes in the *pfhrp2* and *pfhrp3* genes. Based on this, the aim of the current research was to examine the occurrence of *pfhrp2* and *pfhrp3* gene deletions in suspected *P. falciparum* isolates in malaria-endemic regions in Bangladesh and to determine their implications in the diagnosis and surveillance of malaria.

Methods

Study sites and sample collection

This investigation was carried out in the malaria hyper-endemic district of Bandarban, which is located in the CHT regions and borders Myanmar to the east, India to the north, Rangamati district to the west and Cox's Bazar district to the south. The observation of discrepancies between HRP2 RDTs (negative) and microscopy (positive) during the routine process of screening malaria suspected cases prompted the initiation of sample collection.

In mid-2023, the first such discordant infections were observed when two patients in Alikadam upazila (sub-district) with febrile illness and malaria-compatible symptoms but negative RDT results were identified at the point of care. Although the initial RDT result was negative, light microscopy of blood smears showed the presence of *P. falciparum* parasites, and subsequent real-time PCR confirmation identified both cases as likewise. Those two samples, collected in August and September 2023, were the first documented cases of mRDT-negative but microscopy-confirmed *P. falciparum* infections in Bandarban almost ten years after the initial case report of *pfhrp2/3* deletions causing false negative RDT in Sylhet in 2013 [22], which sparked new concern about potential emergence of diagnostic discordance in Bangladesh.

Surprisingly, no further discordant samples were reported during the remainder of 2023 or in 2024. However, in the beginning of May 2025, reports of RDT-negative but microscopy-positive cases became more frequent. In addition to Alikadam, one discordant infection was detected in Rowangchhari upazila as well, indicating that the phenomenon was not geographically restricted to a single locality. At the

same time, NMEP initiated referrals of suspect samples to the Emerging Infections & Parasitology Laboratory (EIPL) at icddr,b for further investigation. The EIPL is the only Bangladeshi lab participating in the "WHO External Quality Assurance (EQA) Assessment Scheme for malaria nucleic acid amplification techniques (NAAT) and detection of molecular markers (MM) of antimalarial drug resistance". The referred samples originated from field-based malaria surveillance activities conducted by NMEP in Lama and Thanchi upazilas, where symptomatic patients had tested negative by RDT but demonstrated detectable parasitaemia on microscopy. Molecular testing further confirmed these infections as *P. falciparum*-positive. A total of ten discordant blood samples were referred by NMEP in EDTA tubes. At the same nine additional discordant samples were referred to icddr,b field team from Alikadam between May to December 2025.

Overall, a total of 22 clinical *P. falciparum* samples were included for molecular investigation in this study. Thus, samples were obtained through two complementary pathways: (i) direct collection through laboratory-based investigation of discordant diagnostic cases identified at different study sites (n = 12), and (ii) a snowball referral mechanism through the national surveillance system, whereby NMEP submitted additional RDT-negative suspected falciparum infections for molecular characterization (n = 10).

Plasmodium infection detection by RDTs

All enrolled patients presented with febrile illness and clinical features suggestive of malaria. Following standard aseptic procedures and universal biosafety precautions, approximately 3 mL of venous blood was collected from each patient into a sterile K_3-EDTA anticoagulant tube for diagnostic testing and subsequent molecular analyses. Initial malaria screening was performed at peripheral health facilities in the field, where RDTs serve as the primary tool for point-of-care detection of Plasmodium infection. All suspected febrile patients were first tested using routine PfHRP2 and pLDH based RDT according to the manufacturers' instructions. Following referral to the EIPL, all samples were subsequently re-tested under controlled laboratory conditions using five commercially available *P. falciparum*-specific HRP2-based RDT platforms- CareStart™ Malaria Pf/Pv (HRP2/pLDH) Ag RDT (Access Bio, Somerset, NJ, USA), Bioline™ Malaria Ag Pf/Pv (Abbott Rapid Diagnostics, Lake Bluff, IL, USA), FalciVax (Zephyr Biomedicals, Goa, India), qDetect Malaria Pf/Pv Antigen Test (OMC Healthcare., Dhaka, Bangladesh), and One Step Malaria (Pf/Pv) Tri-line Test (InTec Products Inc., Xiamen, China).

Determination of parasitaemia by light microscopy

Microscopic examination was performed to confirm malaria infection and quantify *P. falciparum* parasitaemia following the WHO standard guidelines [23]. Initial microscopy was performed at the respective field sites in Bandarban by trained microscopists using Giemsa-stained thick and/or thin blood smears prepared from venous blood. These slides were then transported to the icddr,b laboratory at the Mohakhali campus, Dhaka for quality assurance and independent re-evaluation. The second expert microscopist re-read each slide for parasite species determination and parasite density estimation.

Where there was a discrepancy between the first and second readings—such as disagreement in parasite species determination or parasitaemia quantification—a third, senior microscopist reviewed the slide and gave a final opinion. Crucially, all microscopists were blinded to the results of each other to avoid bias and ensure accurate diagnosis.

DNA extraction and confirmation of Plasmodium infection by SYBR Green PCR

The genomic DNA used for molecular genotyping was extracted from 200µL of EDTA-anticoagulated whole blood using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer protocol and purified DNA was eluted in 50µL of elution buffer.

Species-specific SYBR Green-based PCR assays were conducted to confirm malaria infection and differentiate between the four human-infecting Plasmodium species (*P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*) as previously described with minor adaptation [24]. In brief, 0.7 µL of 10 µM forward and reverse primer (each), 10 µL of iTaq Universal SYBR Green Supermix (Bio-Rad), and 5.6 µL of nuclease-free water were added to each reaction to make the final master mix volume of 17 µL. The 3 µL of extracted DNA of each sample was added to this to obtain a final reaction volume of 20 µL. Thermal cycling was started with an initial denaturation period of 10 min at 95°C, 35 cycles of amplification with denaturation at 95°C, annealing at 57°C, and extension at 61°C. Each elongation phase was followed by recording of fluorescence signals to facilitate real-time identification of amplification products.

Conventional PCR-based amplification of the pfhrp2/pfhrp3 genes and their flanking genes

DNA samples from diagnostically discordant *P. falciparum* infections, which were negative by HRP2-based RDTs but positive by microscopy and RT-PCR, were analyzed to evaluate deletions in the *pfhrp2* and *pfhrp3* genes. Amplification of the target genes was performed following WHO–recommended protocols for *pfhrp2/pfhrp3* deletion surveillance [25], with minor adaptations.

All 22 samples were first screened to assess the presence of *pfhrp2* and *pfhrp3* by end-point PCR amplification of exon-2 of both genes, encoding the antigenic repeat region recognized by HRP2-based RDTs. PCR tests were then performed on flanking loci of the target genes, such as MAL7P1_230 (upstream of *pfhrp2*), MAL7P1-228 (downstream of *pfhrp2*), MAL13P1-485 (upstream of *pfhrp3*), and MAL13P1-475 (downstream of *pfhrp3*) The presence of these flanking regions made it possible to distinguish between partial and complete deletions of the genes.

The products of PCR amplification were separated using 2% agarose gel electrophoresis. The PCR amplicons were loaded in the presence of a 100 bp DNA ladder (4–5 µL) as a molecular size marker. Electrophoresis was performed at 85–95 V over a period of 75 minutes and DNA bands were visualized using a UV transilluminator. The occurrence or lack of particular amplicons was identified according to the anticipated fragment sizes (750 bp for PfHRP2 and 700 bp for PfHRP3) in comparison to the molecular ladder. Each PCR run contained reference lab strains to confirm assay performance. *P. falciparum* 3D7 strain was used as a positive control of *pfhrp2* and *pfhrp3*. Samples were classified as

having a gene deletion only when amplification of the target gene failed consistently across repeated PCR attempts in the presence of successful amplification of control loci.

Probe-based PCR for pfhrp2 and pfhrp3 deletion

Probe-based real-time PCR assays were conducted to determine the deletion status of *pfhrp2* and *pfhrp3* with primers and hydrolysis probes based on the multiplex qPCR assay described elsewhere [26] to complement our conventional PCR-based findings. A duplex reaction with *pfhrp2* and *pfhrp3* was set up in a 25 µL final volume, which contained 12.5 µL of Taqman Fast Advanced Master Mix (2X), 0.3 µL of forward and reverse primers of both *pfhrp2* and *pfhrp3*, 0.25 µL of each corresponding probe, nuclease-free water, and 5 µL of extracted DNA. Simultaneously, another real-time PCR reaction was conducted on the human β-tubulin (HumTuBB) target as an internal control to ensure the integrity of DNA and amplification competence. This reaction was also set up in a final volume of 25 µL using the same master mix, with designated primers and probe added at the concentrations described above and the template DNA adjusted accordingly.

Amplification was carried out under the following cycling conditions: an initial denaturation at 95°C for 3 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s. Fluorescence signals were acquired in the FAM, HEX, and Cy5 channels for *pfhrp2*, *pfhrp3*, and HumTuBB, respectively. Because all isolates had been previously confirmed as *P. falciparum* by species-specific SYBR-Green PCR, failure of *pfhrp2* and/or *pfhrp3* amplification in the presence of successful HumTuBB amplification was interpreted as evidence of deletion of the corresponding target gene.

Results

Demographic characteristics of the study population

Among the 22 discordant *P. falciparum* samples tested, 14 (63.6%) were drawn from male patients and the remaining 8 (36.4%) were from female patients. According to age, the majority of cases were reported among individuals aged ≥ 15 years (n = 15, 68.2%), followed by six cases among individuals aged 4–15 years (27.3%) and a single case from children aged 1–4 years (4.5%). Geographically, 50% of the isolates were originated from Alikadam (n = 11), followed by Thanchi (n = 07; 31.8%), Lama (n = 03; 13.6%), and Rowanchhari (n = 1;4.5%) (Fig. 1).

[Figure 1 to be here]

Distribution of parasite densities and corresponding Ct values

Our microscopy data revealed a substantial variation in parasite densities across isolates, spanning from 440 to 215,000 parasites/µL. All isolates were confirmed as *P. falciparum* mono infection by RT-PCR with Ct values ranged from approximately 14 to 25. As illustrated in Fig. 2, higher parasite densities generally corresponded to lower Ct values, reflecting the expected inverse relationship between parasitemia and amplification threshold. However, some isolates had comparatively low microscopy-based parasite counts even though Ct values were low indicative of high parasite burden (Supplementary Data Table 1: Sample information).

[Figure 2 to be here]

Deletion status of pfhrp2/3 genes and their corresponding flanking regions

All *P. falciparum* isolates exhibited the deletion of *pfhrp2* gene (Table 1). Concurrent deletion of *pfhrp3* was identified in 20 samples, reflecting a high predominance (90.9%) of double *pfhrp2/ pfhrp3* deletion (Supplementary Fig. 1). Conventional PCR analysis of flanking regions revealed a very conserved deletion pattern at the *pfhrp2* locus, with a consistent loss of the upstream marker (MAL7P1_230) and no loss of the downstream region (MAL7P1_228), suggesting a uniform deletion boundary at this locus across the sample set. In contrast, the *pfhrp3* locus displayed greater variability, while the downstream marker (MAL13P1_475) was deleted in all isolates, the upstream region (MAL13P1_485) was retained. Additionally, probe-based multiplex qPCR was performed to validate the findings from conventional PCR. The qPCR results were concordant with the conventional PCR data, confirming *pfhrp2* gene deletion in all 22 samples and *pfhrp3* gene deletion in 20 samples (Supplementary Fig. 2). Altogether, these findings highlight the predominance of dual-gene deletions and suggest locus-specific differences in the genomic structure of deletion events.

[Table 1 to be here]

Discussion

Our findings confirm the presence of *pfhrp2* and *pfhrp3* deleted parasites *in* hyper-endemic malaria regions in Bangladesh. A predominance of double-deletion parasites (~ 91%) indicates a selective pressure on these parasites, possibly due to the widespread use of RDTs in endemic regions, which were introduced in Bangladesh in 2007 [27]. The uniform deletion pattern at the *pfhrp2* locus and variability at the *pfhrp3* locus are in agreement with previous reports of different level of stability of these genomic regions [28–30]., The implication is clear and evident that current PfHRP2-based RDTs are leading to false negative results and surveillance must be urgently conducted to determine the population prevalence in the CHT region. A high prevalence of deletions will generate a diagnostic blind spot that would enable ongoing transmission and threaten malaria elimination goal in Bangladesh. Besides, *hrp3* was retained in two *hrp2*-deleted isolates. Both samples remained RDT-negative despite detectable but low parasitaemia (440 and 1200 parasites/ µL), suggesting that HRP3 cross-reactivity was insufficient to generate detectable antigen levels of parasitaemia. However, previous studies have reported otherwise

that PfHRP3 is generally expressed at lower levels than PfHRP2, pfHRP2-based RDTs vary in their ability to detect PfHRP3 due to differences in antibody specificity and epitope recognition and they require parasitemia ≥ 1000 parasites/μL for PfHRP3 to generate positive results in the absence of PfHRP2 [31, 32].

When *pfhrp2/3* deletions were first reported in Peru in 2010 [5], it was initially considered a localized phenomenon. However, over the past decade, *pfhrp2/3*-deleted parasites have been identified across multiple continents. In Latin America, some Amazonian regions have documented deletion rates as high as 40% [17], while African studies have reported moderate levels in West and Central Africa and much higher frequencies in the Horn of Africa [12, 18, 33]. Prior reports from SA regions have generally documented low to moderate frequencies of *pfhrp2/3* deletions. Large multi-state surveillance studies from India reported overall *pfhrp2* deletion rates of only 0.4–2.4%, with *pfhrp2/pfhrp3* double deletions remaining uncommon [6, 34]. Even in border regions of Myanmar, deletion prevalence has typically been reported at single-digit percentages [35, 36]. However, a recent study in Odisha, India showed that 61.6% of RDT-negative but PCR-positive *P. falciparum* subpatent infections, including both febrile and afebrile individuals, had *pfhrp2* deletions [3]. Bangladesh can now be mapped as a possible high-prevalence deletion zone within SA regions, according to our finding. Prior to this, only a single sporadic case was known and reported from our EIPL at icddr,b in Bangladesh [22].

We observed that the deletions in our study samples encompassed neighboring genes, particularly *pfhrp2* together with its upstream gene and *pfhrp3* with its downstream gene. This pattern mirrored what was reported in Ethiopia [37], India [3], Brazil and Bolivian regions [38]. Although interestingly in other study conducted in Ethiopia [33], the deletion of *pfhrp2* tended to extend downstream in 22% of cases and upstream in only 4%. While the precise boundaries of deletions may vary between studies, accumulating evidence indicates that *pfhrp2/3* loss commonly occurs as part of larger sub-telomeric segment deletions rather than isolated gene excision [5, 39]. Conventional PCR-based method, which amplify specific regions of the gene, may not be able to detect smaller sequence changes or in-frame mutations that do not affect primer-binding. These could potentially affect antigen expression or alter epitope structure, and thereby affecting RDT detection [13, 40]. On the other hand, full-length *pfhrp2* gene deletions lead to loss of HRP2 expression and are known to be a major cause of false-negative HRP2-based RDT [41].

The key finding of our study was that the current PfHRP2-based RDTs mayno longer be reliable as a stand-alone diagnostic test in malaria endemic regions of Bangladesh and surveillance to determine the scope and scale of the problem is urgently needed. Our findings revealed a staggering rate of *pfhrp2/3* deletions in samples from individuals with discordant diagnostic results – suggesting this method is reliable for detecting *pfhrp2/3* deletions. These false negative results would be highly risky as patients could develop severe malaria or transmit the disease via gametocytes [42, 43], thereby undermining malaria control. WHO has also cautioned that false-negative RDTs due to *pfhrp2* deletions can be life-threatening and can lead to progression of infection or transmission [44]. Several countries like Eritrea, Djibouti and Ethiopia have switched to non-PfHRP2-based RDTs following surveys indicating that they

prevalences of false negative HRP2 RDTs due to *pfhrp2/3* deletions far exceeded 5% threshold[12]. A similar policy should be considered in Bangladesh should the results of a survey in Bandarban support it. The PfLDH-based RDTs could be a viable alternative as they are able to detect *P. falciparum* regardless of *pfhrp2* deletion. Although historically, PfLDH-based assays are typically less sensitive than HRP2-based RDTs at low parasitaemia and may fail to detect low-density infections, WHO recently prequalified 3 PfLDH based RDTs including a PfLDH-PvLDH RDT [17, 45]. In the pipeline, there are new RDTs that combine both PfHRP2 and PfLDH targets on the same test line so they are able to detect both HRP2 expressing and non-expressing *P. falciparum* parasites to a high standard, but such tests are generally more expensive and therefore the cost-effectiveness of switching must be carefully considered. It is also important to highlight the importance of microscopy and PCR. Microscopy remains the reference standard for clinical malaria in a few upazila health complexes in CHT regions that was critical in confirming cases that RDTs missed. Moreover, maintaining microscopy expertise is challenging and labor-intensive [17]. PCR is the most sensitive and can definitively detect *pfhrp2/3*-deleted infections (as it targets DNA, not antigen), but is not feasible as routine and point-of-care diagnosis in rural and hard-to-reach settings. Ultimately, any large-scale diagnostic policy shift in Bangladesh must be guided by careful evaluation of cost, sensitivity, operational feasibility and field performance to ensure sustained progress toward malaria elimination.

We acknowledge several limitations in our study. First, the sample size (N = 22) is small, and not a random sample of the population; therefore, it is not possible to estimate the prevalence of *pfhrp2/3* deletions causing false negative RDTs. Second, we only sampled one district (Bandarban). The situation in other endemic districts of Bangladesh remains to be formally surveyed. Third, we did not perform any functional HRP2 antigen quantification. However, of the 22 samples, 20 were sent to the -National Institute of Malaria Research (ICMR), New Delhi, India, for external validation. The results showed complete concordance for *pfhrp2* deletion, while discrepancies were observed in one sample for *pfhrp3* deletion.

Following our findings, NMEP has instructed to test all suspected patients by microscopy whenever possible and refer the suspected patients to nearby microscopy center where the facility is not available. NMEP has also procured the BIOCREDIT Malaria Ag Pf/Pv (pLDH/pLDH) assay (Rapigen, Republic of Korea) for use in highly endemic areas of CHT. Furthermore, NMEP has initiated a survey in ten endemic upazilas of Bandarban and Rangamati Districts, following the WHO-recommended protocol [25]. Should this survey reveal that the 5% deletion threshold is exceeded, NMEP has committed to transitioning from HRP2-based RDTs to pLDH-based RDTs for patient diagnosis.

Conclusion

This study shows a high frequency of deletion of the *pfhrp2* and *pfhrp3* genes in *P. falciparum* parasites in samples taken from suspected malaria cases with discordant results (HRP2-RDT negative/microscopy positive) from Bangladesh, which could be compromising the accuracy of HRP2-based RDT. These findings call for an urgent survey to determine the prevalence of *pfhrp2/3* causing

false negative RDT results in the population and subsequent policy adjustment in diagnostic strategies. Sustained progress in malaria control requires timely implementation of new diagnostic tests, such as pLDH-based assays, and ongoing surveillance of parasite genotypes. The Bangladesh case highlights the need for other endemic countries to proactively conduct surveillance and remain vigilant to ensure that diagnostic measures are integrated with therapeutic and vector control measures to prevent the silent transmission of gene-deleted parasites. Through appropriate measures, the consequences of *pfhrp2/3* deletions can be minimized, preserving recent progress in malaria control.

Declarations

Ethics approval and consent to participate

The samples collected from icddr,b projects were approved by the institutional Ethical Review Committee. Written informed consent was obtained from all participants before data collection. Written informed consent was obtained from all participants or their legal guardians prior to enrolment, and, in addition, written assent was collected from all minors above the age of 11 years. Permission for further use of the samples was also obtained from the study participants in the corresponding consent and assent forms. Additional samples obtained from the NMEP surveillance system and followed by molecular investigation for *hrp2/3* mutation were conducted under a separate protocol approved by the Institutional Review Board of Dev Care Foundation.

Consent for publication

Not applicable

Availability of data and materials

The de-identified data and materials underlying this research are available upon reasonable request from the corresponding author (MSA).

Competing interest

The authors have no competing interests to disclose.

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Author Contributions

MSA, MMR (Md Mushfiqur Rahman), AKN, SKD, MHR, JC and MHR conceived and designed the study. AKN, MMR (Md Mosiqure Rahaman), NNF, and AH collected the data. AH, ATT, and MMI conducted the laboratory investigation. MSA and AH contributed to the data analysis and interpretation. MSA, AKN, JC

and MHR supervised the study. MSA and AH drafted the manuscript. All the authors have read and approved the final manuscript**.**

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Table 1

Table 1 is available in the Supplementary Files section.

Figures

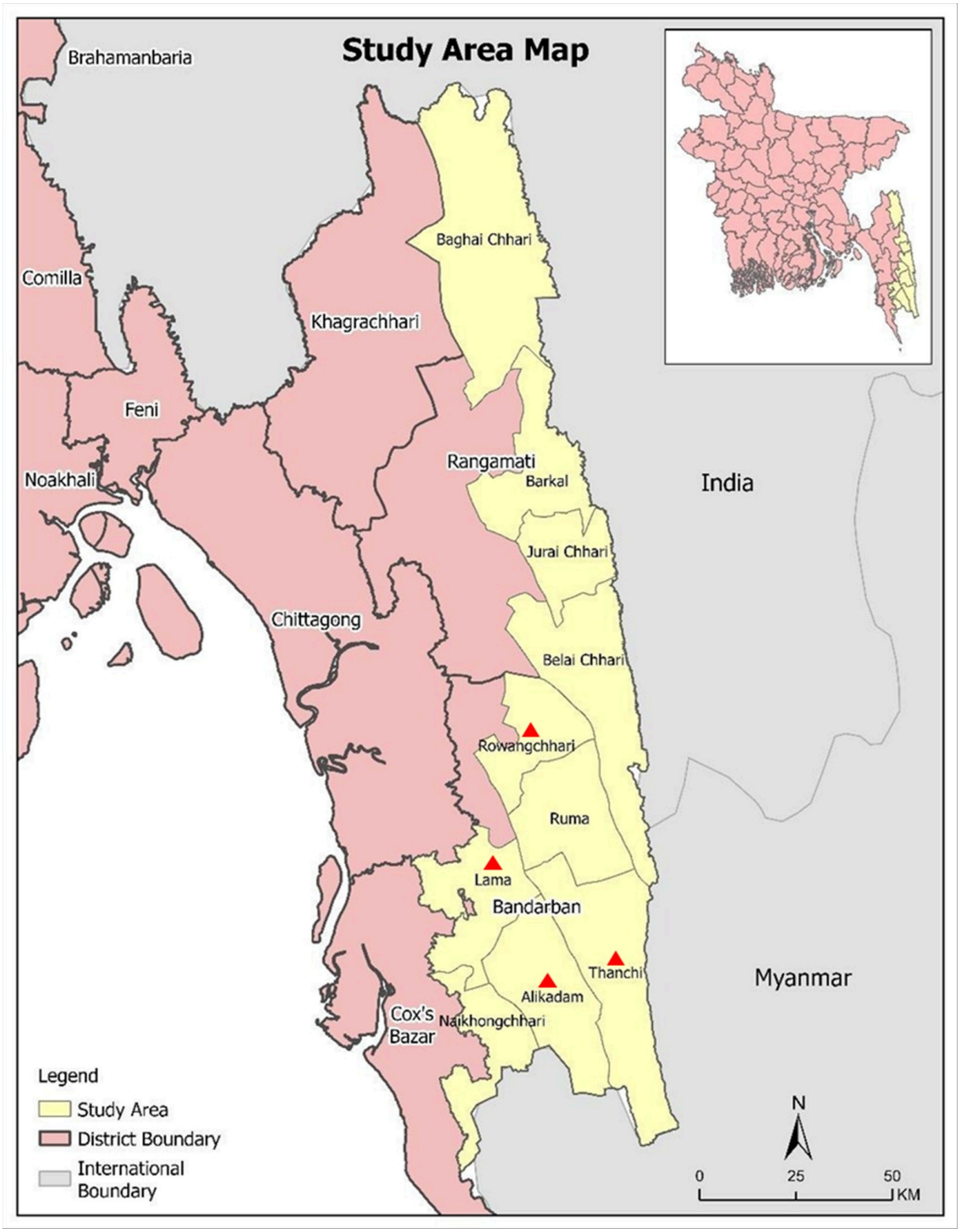


Figure 1

Study area map of Bandarban District showing the locations of sampling sites (red triangles).

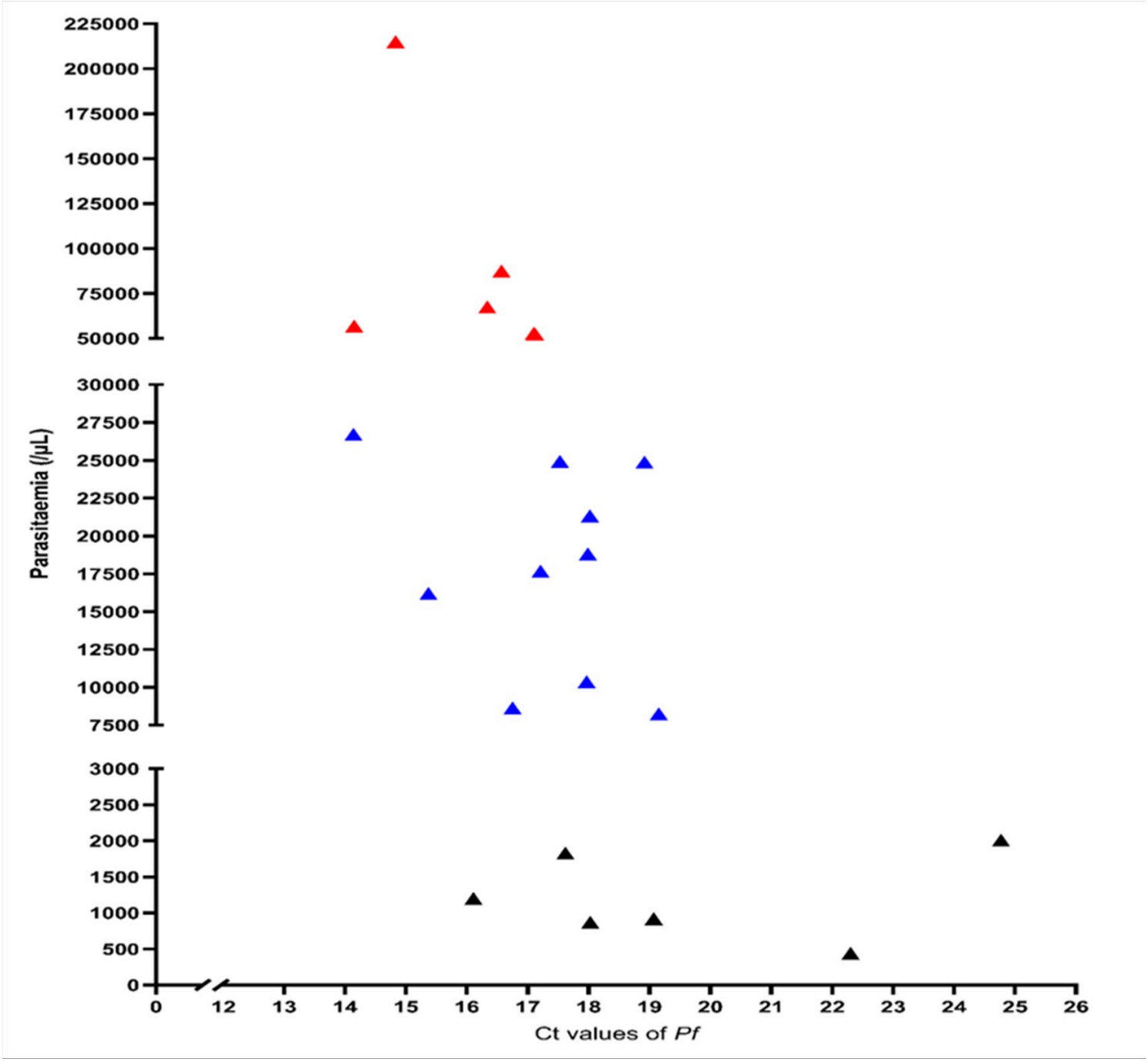


Figure 2

Parasitemia distribution in relation to Ct values among RT-PCR confirmed *P. falciparum* isolates.

Supplementary Files

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- SupplementaryData.docx
- Table1.docx